EXHIBIT 6

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ORIGINAL ARTICLE

Effects of Acetyl Salicylic Acid Therapy on an Experimental Thrombosis Induced by Laser Beam

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Abstract

Aspirin inhibits the synthesis of both platelet and vascular arachidonic acid metabolism which have opposite effects on platelet functions. The rationale for its clinical use as an antithrombotic drug has therefore been questioned. Therefore, we investigated the effects of acetylsalicylic acid (ASA) at 100 mg/kg on an experimental thrombosis induced by laser beams using different groups of rats that were previously treated with the same dose (100 mg/kg), according to the delay between the first and second injections. A partial occlusion was induced by laser beams in the rat mesenteric microvessels (15–25 m). The thrombus formed within seconds after the laser lesion; both it and the embolization which began within minutes after, were continuously accounted. Experiments were done on 11 groups of 5 animals each: 45 rats received a first injection of ASA at j_0 and a second injection 30 minutes before thrombosis induction at j_0+x (x=2, 4, 6, 8, 9, 10, 12, 14 and 16 days). Different groups are defined according to the x value. The rats receiving NaCl 0.9% or a single injection of ASA at 100 mg/kg (n=5) 30 minutes before thrombosis induction were used as control (Group I) and reference group (Group II) respectively. In this study, ASA treatment showed two types of results. The administration of ASA (100 mg/kg) 30 minutes before laser-induced thrombosis prevented thrombus formation. In the same way, ASA injected to rats already treated with the same dose 2 or 4 day later also demonstrated a potent antithrombotic effect. The same trends were observed with animals receiving the second injection (100 mg ASA) at j_{0+8}, j_{0+12}, j_{0+14}, and j_{0+16}. However, when injected to rats at j_{0+6} and at j_{0+10}, ASA did not shown any effects on thrombus formation compared to the control ($p \geq 0.05$). The same phases of ASA action were observed on the induced hemorrhagic time. The antithrombotic effects of the later second injection of ASA (100 mg/kg) were neutralized in rats previously receiving the same dose of this drug. This phenomenon seems to be periodic and is of great importance for the observance of ASA treatment.

Key Words: Thrombosis; Aspirin; Neutralization; Hemorrhage

Acetylsalicylic acid (ASA) is a well known antiaggregative agent used in humans at varying doses to prevent arterial thrombosis, cerebral stroke or myocardial infarction and to reduce the risk of death in patients.

The half-life of ASA in the plasma in vivo is about 15–27 minutes [1,2]. The deacylated product—salicylate—is further metabolized into glucuronic acid and glycine conjugate and, to a minor degree, to dihydroxyamids. The half-life of salicylate is about 2–3 hours [1].

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The absorption kinetic of ASA depends on the galenic form: absorption is promoted when aqueous solutions are administered [2,3].

Bochner et al. [2] have reported that ASA was still detectable for up to 16 hours in the plasma of healthy volunteers who had ingested a single 100 mg dose of ASA.

ASA acts pharmacologically as a potent noncompetitive inhibitor of platelet and endothelial cyclooxygenase through permanent acetylation [4]. The dose-related selectivity of ASA has been ascribed to the ability of endothelial cells to synthesize the novo new enzyme in contrast to platelets which are anucleated [5], as well as the presystemic inhibition of platelets [6].

In vivo and ex vivo studies have shown that administration of high dose ASA (higher than 300 mg/day) inhibits either thromboxane or prostacyclin synthesis. In view of the TXA_2-PGI_2 balance theory [7], selective impairment of vascular cyclooxygenase has been attempted on the assumption that intact prostacyclin production might enhance the antithrombotic efficacy of the drug.

More and more reports show the similar efficiency of the much-tolerated low dose ASA (less than 100 mg/day) [8–15]. Recently, total inhibition of platelet TXA_2 synthesis and preservation of PGI_2 release by endothelial cells have been reported in a trial study [16] using 30 mg ASA/day. Toivanen et al. [17] have obtained the same results with as low as 1 mg/day. The therapeutic benefit of low-dose ASA versus high-dose ASA has yet to be noted, although a greater gastric and renal tolerance has been noted [18,19]: ASA at low or higher doses enhances the bleeding risk in man.

When ASA is present in the microcirculation at doses <1 mg/mL, it showed thromboembolic properties [20,21]. This phenomenon might have relevance in vivo, such as at the end of ASA therapy or after ASA administration during the postoperative period. Another study [22] showed that the subcutaneous administration of the combination of 100 mg/kg and 10^{-30} mg/kg permitted the parameters studied to come back to the control value. So, the variation of the effects of ASA according its plasma concentration, anti- or prothrombotic properties, might limit its therapeutic benefit.

In this experiment, rats were treated with a first single dose of ASA (100 mg/kg). After variable delays according to the groups, rats received a second single dose of ASA (100 mg/kg). The 100 mg/kg of ASA was used because it is the most effective dose in our experimental model of induced thrombosis by laser injuries. Therefore, the aim of this investigation was to analyze the variability of antithrombotic action of a second treatment with ASA and to verify if the first treatment with ASA could modify the action of its second administration, according to the delay between the two treatments.

1. Materials and Methods

1.1. Compounds

The drugs used were aspirin (Aspegic, Laboratoires Synthelabo, France) and saline solution of NaCl 0.9%.

1.2. Experimental Procedure

Male Wistar rats weighing 250–300 g were provided by Depré Center (Saint-Doulchard, France) and were used after 8 days of stabilisation. After administering an anaesthesia with 250 mg/kg of thiopental sodium, a median laparotomy was performed. The intestinal loop was placed on the microscope table and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France). The dynamic course of thrombus formation was continuously monitored and recorded by placing the laser beam (wavelength used=514.5nm) coaxially into the inverted light beam path of a microscope (Axiovert, Zeiss, France). Microscopic images were recorded by a video tape recorder through a video camera (DX L107, colour camera CDD) and were monitored on a television screen (Trinitron colour video monitor, PVM 144 2QM, Sony France). A schematic diagram of the apparatus used has been previously described [23]. Arterioles between 15–25 m diameter were experimented. Each rat underwent two procedures of 10 minutes' duration. Three parameters were assessed during each procedure:

1. The number of laser pulses required to induce a thrombus formation (which has a minimum size of at least one-fourth of the vessel diameter). When no visible thrombus occurred after the first shot, second, third, and fourth laser shots were applied to the vessel

at the same site as the first one (at 1, 5 and 8 minutes, respectively).
2. The number of platelet emboli removed (from the thrombus) by blood flow was measured.
3. The duration of embolization, defined as the time between the first and the last emboli occurring during a ten-minute period of observation.

1.3. Protocol

Rats were randomly assigned into 11 groups of 5 animals each. 45 rats received a first injection of ASA at j_0 and a second injection 1/2 hour before thrombosis induction at j_0+x (x=2, 4, 6, 8, 9, 10, 12, 14 and 16 days). Different groups were defined according to the x value. The rats receiving NaCl 0.9% or a single injection of ASA at 100 mg/kg (n=5) 30 minutes before thrombosis induction were used as control (Group I) and group of reference (Group II), respectively. All the solutions were injected subcutaneously.

1. Group I (n=5): control group. Subcutaneous injection of Na Cl 0,9%.
2. Group II (n=5). Subcutaneous injection of a single dose of ASA (100mg/Kg) 30 minutes before thrombosis induction. Second administration of ASA 100 mg/kg at T_0+2 days:

T_0=First subcutaneous ASA administration to 45 rats (100 mg/kg)

1. Group III (n=5): Second administration of ASA 100 mg/kg at T_0+2 days:
2. Group IV (n=5): Second administration of ASA 100 mg/kg at T_0+4 days
3. Group V (n=5): Second administration of ASA 100 mg/kg at T_0+6 days
4. Group VI (n=5): Second administration of ASA 100 mg/kg at T_0+8 days
5. Group VII (n=5): Second administration of ASA 100 mg/kg at T_0+9 days
6. Group VIII (n=5): Second administration of ASA 100 mg/kg at T_0+10 days
7. Group IX (n=5): Second administration of ASA 100 mg/kg at T_0+12 days
8. Group X (n=5): Second administration of ASA 100 mg/kg at T_0+14 days
9. Group XI (n=5): Second administration at T_0+16 days

Groups are defined as a function of the delay between the first and the second subcutaneous injection of ASA at 100 mg/kg.

2. Biological Analysis

2.1. Platelet Aggregation Study

Platelet aggregation according to the method of Cardinal et Flower [24] was performed on a Chrono Log 500 VS aggregometer (Coultronics, Margency, France) on the whole blood obtained from the rat after laser experimentation. Platelet aggregation was induced by ADP final concentration 5 μM (Laboratoire Diagnostica Stago, France).

Two parameters were determined:

1. Impedance representing the maximum amplitude of aggregation expressed in ohms.
2. Velocity of aggregation expressed in ohms/min.

2.2. Coagulation Tests

At the end of each experiment, blood was collected over 3.8% sodium citrate (9 vol. blood/1 vol. citrate) by cardiac puncture and centrifuged for 20 minutes at 2000 rpm to obtain Platelet Rich Plasma (PRP). Additional centrifugation allowed Platelet Poor Plasma (PPP) to be collected.

Activated Partial Thromboplastin Time (APTT) was performed with an ACL™ (Automated Coagulation Laboratory), which permits the determination of APTT by the automatic addition of cephaline and $CaCl_2$ to the plasma. The intrinsic factors of coagulation are activated by the ellagic acid on an extract of bovine cerebral tissue, substitute of platelet factor III. The coagulation is induced by the addition of $CaCl_2$ (Instrumentation Laboratory, Paris-France).

Prothrombin Time (pTT) and Fibrinogen were performed with an ACL™ (Automated Coagulation Laboratory), which permits the simultaneous determination of the pTT and fibrinogen by using only the Ca-Thromboplastine (Ca-Thromboplastine high analytic sensibility: lyophilized extract of rabbit cerebral tissue from Instrumentation Laboratory, Paris-France).

2.3. Statistical Analysis

Statistical analysis was carried out on PCSM® software (Deltasoft, France). Results were expressed as the mean±standard deviation. Data were compared using the parametric test t of student be-

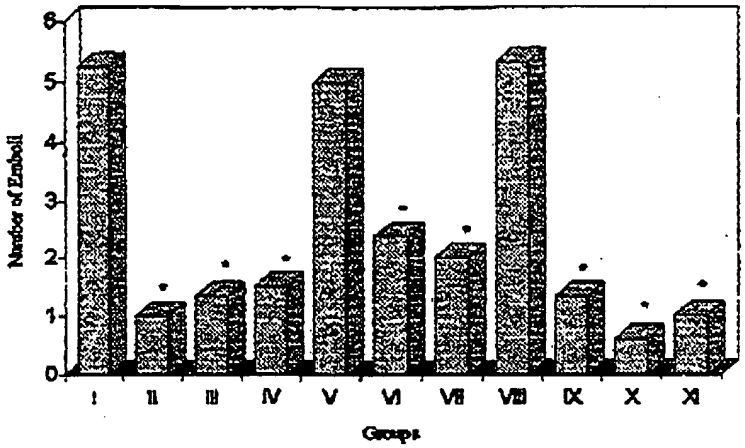

Fig. 1. Effects of ASA treatment on the number of emboli. Thrombosis was induced by endothelial cells destruction by laser beams (120 mW during 1/15 second exposition). Results are expressed as means±1 SD, *: $p \leq 0.05$ (Mann Whitney test).

tween the testing group and the control group. $p<0.05$ was considered significant.

3. Results

3.1. Effects of ASA Treatment on the Thrombus Formation

The administration of a single dose ASA (100 mg/kg) 30 minutes before arterial thrombosis induced by laser beams decreased significantly the number of emboli and the duration of embolization. When ASA was administered to rats previously treated with ASA at the same dose (100 mg/kg) 6 or 10 days before, it did not show differences with control group. For all the other groups, the second injection of ASA demonstrated significant effects. The effects observed in groups previously treated (8 days before) seemed to be less pronounced compared to groups receiving a single dose of ASA 30 minutes before thrombosis induction (Figures 1 and 2).

3.2. Effects of ASA Treatment on the Ex Vivo Platelet Aggregation Induced by ADP

Administered at 100 mg/kg, 30 minutes before experimentation, ASA decreased significantly the amplitude and velocity of the ex vivo platelet aggregation induced by ADP (5 μM final concentration). In contrast, ASA administered at 100 mg/kg to rats pretreated with the same dose 6 days previously increased significantly the amplitude and velocity of platelet aggregation. When the second injection was performed 10 days after the first, no statistical differences were observed when compared to the control group (Table 1).

3.3. Effects of ASA Treatment on the pTT, aPTT and Fibrinogen

Except for rats receiving a second 100 mg/kg ASA administration at J_0+10 days, there were not any significant differences between all the other groups and the control ($p>0.05$). In fact, aPTT for this group was decreased significantly versus the control group (Table 2).

3.4. Effects of ASA Treatment on the Induced Hemorrhagic Time

As shown in Figure 1, a single-dose administration of ASA at 100 mg/kg increased significantly the induced hemorrhagic time. Additional doses of ASA at 100 mg/kg 2, 12, 14 and 16 days after this first injection also increased this parameter, but to less extent. For groups receiving the second injection of ASA 4, 6 and 8 days after the first same dose administration, there was not any significant difference from the control group (Figure 3).

4. Discussion

Thrombotic events usually occur at sites of pathological vascular damage (fissured or ruptured ath-

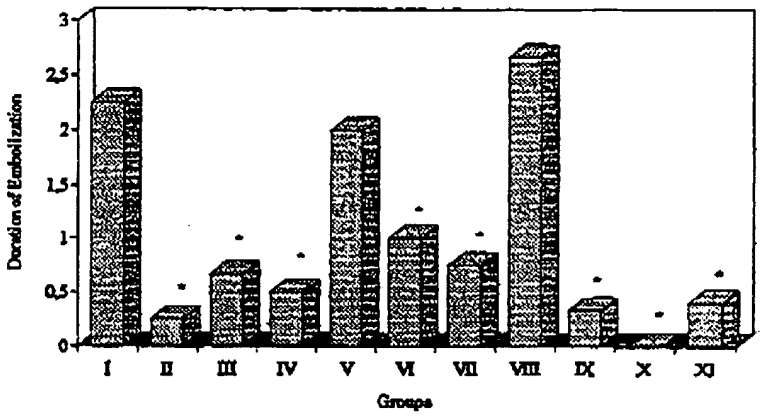

Fig. 2. Effects of ASA treatment on the duration of embolization. Thrombosis was induced by endothelial cells destruction by laser beams (120 mW during 1/15 second exposition). Results are expressed as means±1 SD, *: $p \leq 0.05$ (Mann Whitney test).

erosclerotic plaques). High shear forces created by stenotic lesions in the vessel wall also promote platelet accumulation at these sites. These phenomena play a major role in the pathogenesis of occlusive vascular diseases. Early inhibition of platelet reactivity (before thrombin formation) is essential for prophylaxis of thrombotic events.

Aspirin is effective as an antithrombotic agent. This is especially true for secondary prevention of myocardial infarction [3,25]. It is widely used in clinical practice to prevent arterial thrombosis, cerebral strokes [26] and to reduce the risk of death in patient.

The accepted rationale for ASA treatment of cardiovascular disorders is that the compound inhibits synthesis of TXA_2, an eïcosanoid with pro-aggregatory and vasoconstrictive properties [13]. Such inhibition results in reduction of platelet reactivity as evidenced by prolonged bleeding time and reduced responsiveness to platelet agonist in vitro [27]. When vascular occlusion occurs in a patient treated with ASA, it takes place through a mechanism that overcomes the aspirin-induced platelet functional defect in vivo.

Table 1. Effects of ASA treatment on the Amplitude and Velocity of the *ex vivo* platelet aggregation induced by ADP (5 μM final concentration)

	Amplitude (ohms)	Velocity (ohms/minutes)
Group I (Na Cl 0.9%)	15.75±0.71	16.75±0.96
Group II	7.00±0.01*	10.25±1.50*
Group III	3.33±1.53*	6.67±1.53*
Group IV	12.50±0.71*	11.50±0.71*
Group V	29.00±11.31*	19.50±10.61
Group VI	9.60±2.70*	10.20±2.05*
Group VII	8.25±4.03*	13.50±1.29*
Group VIII	19.00±3.80	15.67±9.87
Group IX	10.00±2.00*	6.00±1.00*
Group X	8.20±4.66*	6.80±3.03*
Group XI	10.20±5.07*	8.60±4.16*

Results are expressed as means±1 SD.
*$p \leq 0.05$, Mann Whitney test.

In the present investigation we studied the effects of ASA treatment in rats' mesenteric vessels in which thrombosis was induced by laser beams. This model was used because of its greatest resemblance to pathological processes resulting from a lesion observed in human disorders. With this model it is possible to obtain restricted lesion of the vessel wall surface. Only a few endothelial cells are damaged. Exposure of the deendothelialized surface to circulating blood initiates platelet and coagulation reactions that result in thrombosis. In the initial step, arterial blood flow can disrupt the hemostatic material that accumulates continuously on the thrombogenic luminal surface, and embolization occurs. Then the thrombus grows rapidly and can obstruct partially or totally the lumen of the vessel.

This experimental study showed that when administered subcutaneously at a dose of 100mg/kg, ASA induced in rats the strongest effect on the number of emboli and duration of embolization. More, the bleeding time was increased compared to the control. The mechanism of this action is duc to an inhibition of cyclooxygenase in platelets.

The high dose of 100 mg/kg ASA was used for

Table 2. Effects of ASA treatment on the pTT, aPTT and the Fibrinogen level

	pTT (sec)	aPTT (sec)	Fibrinogen (g/l)
Group I (Na Cl 0.9%)	17.9±0.54	25.77±0.61	2.18±0.15
Group II	17.07±0.63	23.70±1.09	1.90±0.11
Group III	17.53±0.58	25.2±1.25	2.36±0.15
Group IV	18.60±0.00	20.85±3.18	2.31±0.00
Group V	17.70±0.70	24.65±0.49	2.57±0.17
Group VI	17.90±0.62	24.74±2.29	2.02±0.16
Group VII	18.17±0.88	25.20±1.25	1.86±0.26
Group VIII	17.37±1.19	19.83±5.20*	2.11±0.87
Group IX	18.00±0.26	25.30±0.52	2.23±0.05
Group X	18.20±1.25	23.17±2.03	2.28±0.45
Group XI	17.96±0.96	24.04±0.50	2.31±0.13

Results are expressed as means±1 SD.
* $p \leq 0.05$, Mann Whitney test.

this experimentation according to our later study [23], which demonstrates that the subcutaneous injection of high doses of ASA is more effective against thrombus formation and embolization in the experimental model of laser induced thrombosis by laser injuries.

Results from this study showed that the action of the second administration of ASA is variable according to the delay between the two treatments:

1. When performed two days after the first administration, the treatment with ASA also demonstrates potent antithrombotic activities. In fact, this second administration decreases the number of emboli, reduces the duration of embolization and amplitude of the ex vivo platelet aggregation induced by ADP (5 μM final concentration). Moreover, at T_0+2 days, a second administration of ASA enhances the hemorrhagic effect of ASA.
2. The administration of ASA in the second attention, 6 or 10 days after the first injection at the same dose (100 mg/kg) did not show statistically significant differences from the control group. In this case, ASA probably neutralizes the prothrombotic effect previously observed in rats receiving a single dose ASA at j_0 and undergoing thrombosis induction by laser 6 and 8 days after without second injection [28].
3. When performed at 12, 14 or 16 days after the first injection, ASA in second attention showed its known antithrombotic effect, reducing the number of emboli and the duration of embolization.

It was previously demonstrated that the administration of ASA at 100 mg/kg can prevent thrombus formation induced by endothelial cell damage. But,

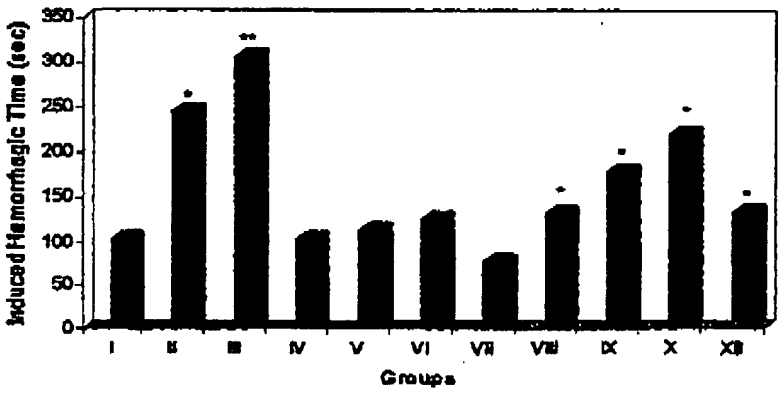

Fig. 3. Effects of ASA treatment on the Induced Hemorrhagic Time (I.H.T). Induced hemorrhagic time was performed on rats 5 minutes before thrombosis induction by laser beams. Results are expressed as means±1 SD, *: $p \leq 0.05$ (Mann Whitney test).

a long time after ASA treatment, this substance provokes an enhancement of thromboembolic complications. So, the effects of ASA may depend on the drug concentration in the microvasculature, pharmacokinitics, and availability as acetyl salicylic in the blood. The effect may be related to an "effect curve": a small concentration of ASA may cause changes in effect on a very steep concentration. As discussed previously [28], this prothrombotic effect of ASA may be due to the generation or the activation of the synthesis of an active metabolite with prothrombotic activity or the inhibition of the synthesis of an agent with antithrombotic activity by ultra low doses of ASA in plasma. So, in this investigation, we postulate that a second injection of ASA at 100 mg/kg, can neutralize this prothrombotic side effect.

Results from this experiment showed that the second injection of ASA at j_0+6 or 10 days neutralize prothrombotic substances activated by ASA at infinitesimal dosages in plasma. At high dosages, ASA induces an antagonization directed against the effect observed some days after the end of ASA therapy. But, the mechanism(s) of these effects remain to be clarified. More investigations must be performed in order to determine an eventual relationship between the dose administered, time and ASA effect. This neutralization might be an interesting tool to prevent thromboembolic complications observed several days after the end of ASA treatment. It is of great importance for the observance of ASA treatment.

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